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COMPLETE SPECIFICATION

Method for the Extraction of Sulphated Polysaccharides

We, RIKER LABORATORIES, INC., a Corporation organised under the Laws of the State of Delaware, United States of America, of 19901 Nordhoff Street, Northridge, State of California, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to an improved method for the extraction of sulphated polysaccharides. The method of the invention will be described with reference to the specific sulphated polysaccharide heparin. Other sulphated polysaccharides, for example chitin sulphate, xylan sulphate, chondroitin sulphate and hyaluronic acid sulphate, are closely related to heparin, in that they are capable of forming an anion, and they may be extracted and isolated by the method of the present invention. It will be appreciated that the precise conditions of operation will vary with the nature of the anion of the particular sulphated polysaccharide present.

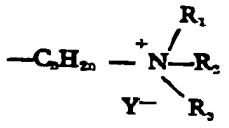
Heparin is a well known naturally occurring anticoagulant which is obtained by extraction from such animal tissues as beef small intestine, beef lung, hog duodenum, hog liver, and hog intestinal mucosa.

All commonly used heparin extraction processes have as an initial step the contacting of the animal tissues with a hot aqueous salt solution which serves to release heparin from the cells of the tissues. The heparin content of the resulting medium is extremely low and extraction techniques heretofore known have not proved sufficiently efficient to ensure high percentage recoveries of the heparin therefrom.

In addition, during the course of any heparin extraction process, heparin fractions of varying degrees of purity may be obtained from which it is desirable to extract more highly purified heparin fractions. An efficient process for effecting such purification has obvious value.

It has now been found that heparin is extracted and isolated in an efficient manner by treating a material containing heparin with a quaternary ammonium anion exchange resin as hereinafter described in an aqueous medium in the presence of an alkali metal, alkaline earth metal or ammonium salt of an acid, separating the resin containing bound heparin from the medium and isolating purified heparin from the separated resin. The method of the present invention may be applied to heparin as present in animal tissues, or in an extract of such tissues, or as obtained at any step in an integrated heparin extraction process.

The present invention provides a method for the extraction of sulphated polysaccharides which comprises treating a material which contains a sulphated polysaccharide in an aqueous medium at a pH between 4 and 10 with a cross-linked copolymer of a mixture of an aromatic monovinyl hydrocarbon and an aromatic divinyl hydrocarbon, said mixture containing 96 to 99.9 mole percent of said monovinyl hydrocarbon and 4 to 0.1 mole percent of said divinyl hydrocarbon, said copolymer bearing on the aromatic nuclei substituent groups of the formula



wherein n is 1 to 4, R_1, R_2 and R_3 are hydrocarbon groups and Y is an anion, said aqueous medium containing a water soluble alkali metal, alkaline earth metal or ammonium salt of an acid, said salt being present in an amount such that at least 50% of the sulphated polysaccharide is bound to the copolymer, and separating the copolymer containing the bound sulphated polysaccharide from the aqueous medium. In the quaternary ammonium anion exchange resin, R_1, R_2 and R_3 are hydrocarbon groups including, for example, lower alkyl, hydroxy-lower alkyl, cycloalkyl, aryl, and

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aralkyl. Y is an exchangeable anion, other than heparin, such as hydroxyl, chloride, sulphate, nitrate, bicarbonate, and acetate. Quaternary ammonium anion exchange resins of the type described above are fully described in British Patent Specification No. 654,706 and are prepared by the techniques described therein.

The resins used in the method of the invention are quaternary ammonium derivatives of copolymers of such aromatic monovinyl hydrocarbons as styrene, o-, m-, and p-methylstyrenes, and o-, m- and p-ethylstyrenes with such aromatic divinyl hydrocarbons as divinyl benzene, divinyl toluenes, divinyl xylenes and divinyl ethylbenzenes. Copolymers of styrene and divinyl benzene are preferred. Where the method of the invention is applied to an aqueous salt solution-animal tissue homogenate as described hereinafter, it is preferred that the copolymer be of a mixture of at least 98 mole percent aromatic monovinyl hydrocarbon and not more than 2 mole percent aromatic divinyl hydrocarbon.

It has been found that styrene-divinyl benzene copolymers containing methylene trimethyl ammonium chloride groups and methylene dimethyl hydroxyethyl ammonium chloride groups aare preferred. Such resins are commercially available under the Registered Trade Marks Dowex-1 and Dowex-2. Such resins having structures formed by copolymerization of mixtures of 98 to 99 mole percent styrene and 2 to 1 mole percent divinylbenzene are particularly effective. A further preferred copolymer is one that contains at least one of the above-defined substituent groups for each 15 aromatic nuclei.

In accordance with the invention, a heparin-containing material is first placed in intimate contact with an aqueous medium, to form a solution or a finely divided uniform suspension. The pH of the medium should be within the range of from 4 to 10 with a pH range of 7 to 9 being particularly effective. It has been found that the aqueous medium must contain a water soluble alkali metal, alkaline earth metal or ammonium salt of an acid, or mixtures of such salts, useful salts including the water soluble sodium, potassium, ammonium, calcium, barium and strontium salts of mineral acids such as hydrochloric, nitric, sulphuric, phosphoric and carbonic and of lower alkyl carboxylic acids such as acetate and propionic. Alkali metal or ammonium nitrates and chlorides have been found to be particularly effective. pH adjustment is most conveniently effected by use of an acid or base having the same anion or cation, respectively, as a salt present in the aqueous medium. Alternately, other acidic or basic substances can be used.

The aqueous medium must contain an effective amount of a salt of the class described hereinabove or mixtures of such salts, that is, a quantity of dissolved salts within the range over which the equilibrium between heparin bound to the resin and heparin remaining in solution ensures that at least 50% of the heparin activity is bound to the resin. It will be appreciated that the range of salt concentrations over which at least 50% of the heparin present in the aqueous medium is taken up by and becomes bound to the resin varies with the particular salt system present, but may readily be determined by a simple test procedure in which a standard heparin solution of known concentration in an aqueous medium containing dissolved salt is stirred with an excess of the quaternary ammonium anion exchange resin to be used in the recovery process at the pH conditions to be used in the process for a period of two hours. The resin is removed and the heparin potency in the supernatant liquid is determined. If the supernatant liquid contains less than 50% of the heparin activity originally present in the standard solution, an effective amount of dissolved salt is present in the aqueous dissolved medium used in the test. With the particularly effective alkali metal and ammonium nitrates and chlorides, it has been found that at least 0.1 mole of dissolved salt per litre of solution should be present. When an alkali metal or ammonium nitrate is used, it is preferred that the salt concentration not exceed one mole per litre, and with alkali metal or ammonium chlorides the dissolved salt concentration should not exceed 1.25 moles per litre to ensure the optimum degree of heparin uptake by the resin.

In carrying out the process of the present invention an excess of the quaternary ammonium anion exchange resin is added to the aqueous salt medium containing crude heparin-containing material in solution or suspension. The mixture is stirred so as to maintain a relatively even dispersion of the resin particles until two consecutive samples of the supernatant liquid upon assay shows substantially equal levels of heparin activity, thus indicating that equilibrium has been attained. If periodic samples of the supernatant liquid as described above indicate the presence of a substantial concentration of heparin in the aqueous medium, additional resin is added to ensure that an excess is present.

Alternately, the aqueous medium can be passed downwardly over a bed of resin in a column. The technique of alternate stirring and settling is generally preferred since it enables one to follow closely the course of the adsorption to ensure substantially complete extraction.

At the conclusion of the adsorption step described above, the resin containing bound heparin is washed and processed for the isolation of heparin therefrom by elution with an aqueous solution of an alkali metal or ammonium salt of a mineral acid, with alkali metal of ammonium nitrates and chlorides being preferred, for example sodium chloride,

potassium chloride, ammonium nitrate, ammonium chloride, sodium nitrate and potassium nitrate. It will be appreciated that the nature of the anion is more important than that of the cation. To facilitate reuse of the resin in subsequent heparin extraction runs, the anion present in the eluting solution should be the same as the anion (Y in above formula) originally present in the quaternary ammonium anion exchange resin. To ensure optimum isolation of heparin, it is desirable that the aqueous salt solution used for elution be of a sufficiently high ionic strength to ensure substantially complete release of heparin from the resin. This concentration varies with the nature of the anion in the salt solution.

It has been found that the use of an aqueous solution of an alkali metal or ammonium nitrate or chloride having a concentration of at least 1.9 moles of dissolved salt per litre of solution and up to saturation is desirable to ensure a high degree of heparin release from the resin to the eluting solution.

In accordance with a preferred embodiment of the invention, significant further purification of heparin can be realised by carrying out at least two elutions at differing salt molarities. The first elution is carried out with an aqueous solution of an alkali metal or ammonium salt of a mineral acid at a salt concentration at which essentially no heparin activity is released from the resin. Again this concentration varies with the nature of the anion. For example, the resin containing bound heparin may be first contacted with a first aqueous eluting solution of an alkali metal or ammonium nitrate at a concentration of from 1 to 1.25 molar or an alkali metal or ammonium chloride at a concentration between 1.25 and 1.6 molar. Under these conditions, the material which is eluted from the resin is essentially devoid of heparin activity. After the contact with a first eluting solution as described above, the resin may be contacted with an aqueous solution of an alkali metal or ammonium nitrate or chloride at a concentration greater than 1.9 molar, thereby releasing the heparin bound to the resin to the eluting solution, without contamination with the inactive material removed by the first solution.

In accordance with a still further embodiment of the invention, the molarity of the salt solution used to release heparin bound to the resin is maintained within the range of 1.9 molar to 2.3 molar. It has been found that by eluting first at a molarity not exceeding 2.3 molar, the material released from the resin by contact thereafter with a salt solution having a concentration of greater than 2.3 molar is devoid of any significant amount of heparin activity. Accordingly, the heparin released from the resin is not contaminated with the material which remains on the resin at this molarity.

The resin remaining after solution, in accordance with the various elution processes described above, is suitable for reuse in subsequent heparin extraction runs without further purification. For optimum results, however, it is desirable to subject the resin to a "clean-up" elution step in which the resin is contacted with an aqueous salt solution of high molarity, preferably in excess of 3 molar, which serves to remove inactive material bound to the resin, thereby to yield a clean resin ideally suited for reuse in subsequent heparin extraction processes.

The elution can also be carried out in a gradient fashion, whereby the resin is contacted with successive small fractions of aqueous solutions of an alkali metal or ammonium salt of a mineral acid of progressively increasing molarities, for example, to at least 1.9 molar. The successive fractions can then be processed separately to isolate therefrom fractions of varying heparin potency. In this manner, heparin fractions can be obtained having potencies of the order of 200 units per milligram.

After contact of the resin containing bound heparin with a salt solution or solutions as described above, the solution or solutions containing heparin are processed for the isolation of the heparin therefrom by conventional procedures, for example, treatment with an organic solvent such as methanol, ethanol or acetone, which results in precipitation of heparin from the solution.

The method of the invention is ideally suited for the extraction of heparin from aqueous salt animal tissue extracts. The slurry of animal tissue and aqueous alkaline salt solution can, for example, be filtered or centrifuged and the resulting filtrate or centrifugate, after pH and salt concentration adjustments, if necessary is then suitable for treatment with the quaternary ammonium anion exchange resin as described hereinabove. Alternately, the slurry can be homogenized and the homogenate treated with resin in accordance with the invention. Inasmuch as effective extraction of heparin from tissues can be obtained in aqueous salt solutions under conditions which are particularly effective for the method of the present invention, it is apparent that with such extraction conditions, treatment with the quaternary ammonium anion exchange resin can be effected conveniently without further pH or salt concentration adjustment. The treatment of a tissue-aqueous extract homogenate with resin is particularly desirable since the step of filtration of the tissue slurry is eliminated. The resin, being in discrete particulate form, readily separates from the homogenate and is treated for the isolation of heparin therefrom as described hereinabove.

The method of the invention is also adapted to the extraction of purified heparin from any heparin-containing material obtained in a heparin extraction process. Where the material

is a solid, it is placed in an aqueous salt solution and the resulting solution is treated with a quaternary ammonium anion exchange resin as described above. Where the material is an aqueous solution, the solution can be readily treated with resin after pH and salt concentration adjustment, if necessary.

The method of the present invention may result in excellent yields of heparin. The quaternary ammonium anion exchange resin is readily recovered from the aqueous medium after the treatment step and after separation of the heparin therefrom is suitable for reuse in subsequent runs.

Following is a description by way of example of methods in accordance with the invention.

EXAMPLE 1

8,200 pounds of hog casing mucosa, 200 gallons of water and 350 pounds of ammonium chloride are added to a jacketed vessel fitted with a stirrer. The pH is adjusted to 9.0 by the addition of ammonia. The mixture is heated with steam while under continuous agitation until the temperature reaches 78° C., at which time coagulation of the proteinaceous material occurs.

A 350 litre aliquot of the above heat coagulated mixture having a pH of 8—8.5 and an ammonium chloride content of 0.5 molar is homogenized twice. To 320 litres of the resulting homogenate (heparin potency = 37 u/ml.) are added 6 litres of Dowex 1—X1 resin. Dowex 1—XI resin is a copolymer of a mixture of 99 mole percent styrene and 1 mole percent divinylbenzene containing on the aromatic nuclei methylene trimethylammonium chloride groups (approximately one such group for each 1.7 aromatic nuclei).

The resulting mixture is stirred overnight and the resin separated after the addition of water to reduce viscosity. The potency of the supernatant homogenate after removal of the resin is 5 heparin units per ml., indicating that the resin has taken up in excess of 85% of the heparin present in the input material.

The separated resin containing bound heparin is contacted with two 12 litre portions of 20% sodium chloride solution (about 3.9 molar). The two solutions are combined and heparin precipitated therefrom with 1.5 volumes of methanol. The resulting precipitate is collected and dried to yield 107 grams of crude heparin having a potency of 76 units per mg. This represents the extraction of 73% of the heparin activity present in the input material and is equivalent to the extraction of 15,500 heparin units per pound of hog mucosa starting material, far in excess of the 9,500—11,000 units/per pound in prior art processes.

The results set forth in the foregoing Example indicate that the method of the present invention may be readily adapted to extract heparin at high yields from a tissue-extracting medium homogenate. The step of filtration of the tissue residue from the extract is eliminated, thus representing a marked simplification in processing techniques.

EXAMPLE 2

A filtered heparin-containing extract at pH 8.0—8.5 assaying 29 units/ml and containing ammonium chloride (0.5 M) is applied to a column containing Dowex 1—X2 resin, identical to the Dowex 1—X1 described in Example 1 except that the copolymer is formed of a mixture of 98 mole percent styrene and 2 mole percent divinylbenzene. Elution with a 25% sodium chloride solution (about 5.1 molar) results in the extraction of 88.3% of the heparin present in the extract.

The resin after the above described elution is carried through two successive runs in extracting heparin from a filtered tissue extract containing heparin. Extractions of heparin are 93 percent and 87 percent respectively, indicating that the resin is adapted for reuse after elution without reduction in yield.

EXAMPLE 3

A quantity of 100 mls. of Dowex 1—X1 resin is placed in a 32 millimetre diameter glass column and 3 litres of a heparin-containing extract (ammonium chloride at 0.5 molar, pH = 8—8.3) is passed downwardly through the column at a constant rate of 40 ml./hour. Thereafter, the column is washed at a rate of 700 ml./hour with 4 litres of 0.5 molar sodium chloride solution at a pH of 8.0.

The resin containing bound heparin is then subjected to elution with a sodium chloride solution at a progressively increasing salt concentration of 0.5 to 2.7 molar. Fractions of effluent were collected, precipitated with methanol, the precipitates weighed and analysed for heparin content with the results as tabulated below:

Fraction	Range of Salt Molarity	Weight of Precipitate (mg)	Heparin Potency (u/mg)	Total Activity (units)	% of Eluated Activity in Fraction
1	0.51 — 1.25	94.6	1.0	95	0.2
2	1.25 — 1.62	149.1	20.8	3,100	6.3
3	1.62 — 1.74	134.0	120.0	16,050	32.7
4	1.74 — 2.02	186.5	138.0	25,500	51.8
5	2.02 — 2.28	41.9	89.0	3,730	7.6
6	2.28 — 2.6	33.0	20.2	665	1.4
				49,140	100.0

The foregoing Example illustrates the results obtained with step-wise or gradient elution in accordance with a preferred embodiment of the invention.

EXAMPLE 4

A series of experiments is carried out to investigate the use of salts other than ammonium chloride in the aqueous heparin-containing medium contacted with quaternary ammonium anion exchange resin in accordance with the invention.

In each experiment, 250 ml. of an aqueous heparin solution (activity = 28.2 u/ml.) at pH 7 and a salt concentration of 0.5 normal is stirred for two hours with 2 ml. of Dowex 1—X2 resin (as described in Example 2). Samples of the supernatant are then taken and assayed.

The results of the experiments are as follows:

Salt	% of Heparin Activity Absorbed by Resin
Sodium Chloride	Greater than 95%
Sodium Nitrate	Greater than 95%
Sodium Phosphate	88%
Sodium Acetate	71%
Ammonium Sulphate	62%

The results reveal that sodium chloride and nitrate are equivalent to ammonium chloride in that essentially quantitative adsorption of heparin on the resin occurs. With the other salts, substantial heparin absorption occurs.

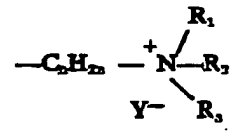
The invention also includes within its scope quaternary ammonium salts of heparin in which the cationic portion is the cation of a quaternary ammonium anion exchange resin as described above and the anion is a heparin ion. Such heparin salts are obtained as described above by treatment of a heparin-containing material with the resin and are useful as intermediates in the purification of heparin in accordance with the process aspect of the invention. They may be stored after preparation awaiting future elution to prepare purified heparin.

The invention is not limited to the details of the foregoing examples; for example, the polysaccharide containing material may comprise sulphated polysaccharides other than heparin.

WHAT WE CLAIM IS:—

1. A method for the extraction of sulphated polysaccharides which comprises treating a material which contains a sulphated polysaccharide in an aqueous medium at a pH between 4 and 10 with a cross-linked copolymer of a mixture of an aromatic monovinyl

hydrocarbon and an aromatic divinyl hydrocarbon, said mixture containing 96 to 99.9 mole percent of said monovinyl hydrocarbon and 4 to 0.1 mole percent of said divinyl hydrocarbon, said copolymer bearing on the aromatic nuclei substituent groups of the formula



wherein n is 1 to 4, R_1, R_2 and R_3 are hydrogen groups and Y is an anion, said aqueous medium containing a water soluble alkali metal, alkaline earth metal or ammonium salt of an acid, said salt being present in an amount such that at least 50% of the sulphated polysaccharide is bound to the copolymer, and separating the copolymer containing the bound sulphated polysaccharide from the aqueous medium.

2. A method as claimed in claim 1 wherein said cross-linked copolymer is a copolymer of a mixture of 98 to 99 mole percent styrene and 2 to 1 mole percent divinylbenzene and wherein each substituent group is a methylene trimethyl-ammonium chloride group.

3. A method as claimed in claim 1 wherein said cross-linked copolymer is a copolymer of a mixture of 98 to 99 mole percent styrene and 2 to 1 mole percent divinylbenzene and wherein each substituent group is a methylene dimethyl - 2 - hydroxyethylammonium chloride group.

4. A method as claimed in any one of the preceding claims wherein the pH of the aqueous medium is from 7 to 9.

5. A method as claimed in any one of the preceding claims wherein the aqueous medium contains an alkali metal or ammonium nitrate at a concentration of from 0.1 to 1 molar or an alkali metal or ammonium chloride at a concentration of from 0.1 to 1.25 molar.

6. A method as claimed in any one of the preceding claims wherein the sulphated polysaccharide is isolated from the separated copolymer, to which it is bound, by elution with an aqueous solution of an alkali metal or ammonium salt of a mineral acid at a concentration of at least 1.9 molar.

7. A method as claimed in claim 6 wherein the elution is carried out with an aqueous solution of an alkali metal or ammonium nitrate or chloride.

8. A method as claimed in claim 6 wherein the separated copolymer containing the bound sulphated polysaccharide is contacted, before elution, with an aqueous solution of an alkali metal or ammonium salt of a mineral acid, said salt being present at a concentration at which essentially none of the sulphated polysaccharide is released from the resin.

9. A method as claimed in claim 8 wherein the copolymer containing the bound sulphated polysaccharide is first contacted with an aqueous solution of an alkali metal or ammonium nitrate at a concentration of from 1 to 1.25 molar, or an alkali metal or ammonium chloride at a concentration of from 1.25 to 1.6 molar.

10. A method as claimed in claim 6 wherein the concentration of the eluting solution is from 1.9 to 2.3 molar.

11. A method as claimed in claim 6 wherein the elution is carried out with a plurality of aqueous solutions containing an alkali metal or ammonium salt of a mineral acid and having progressively increasing molarities to at least 1.9 molar.

12. A method as claimed in claim 10 wherein the separated copolymer is finally contacted with an aqueous solution of an alkali metal or ammonium salt of a mineral acid at a concentration of at least 3 molar, thereby yielding a copolymer suitable for reuse.

13. A method as claimed in any one of the preceding claims wherein the sulphated polysaccharide after isolation from the separated copolymer is precipitated from solution by the addition of an organic solvent.

14. A method as claimed in any one of the preceding claims wherein the sulphated polysaccharide is heparin.

15. A method as claimed in any one of the preceding claims wherein the copolymer contains at least one of the defined substituent groups for each 15 aromatic nuclei.

16. A method for the isolation of heparin substantially as described herein with reference to any one of the Examples.

17. A cross-linked copolymer containing bound sulphated polysaccharide when obtained by the method claimed in any one of the preceding claims.

18. Sulphated polysaccharide when isolated from the polysaccharide-containing copolymer claimed in claim 17.

19. A quaternary ammonium heparin salt of a cross-linked copolymer of a mixture of 96 to 99.9 mole percent of an aromatic monovinyl hydrocarbon and 4 to 0.1 mole percent of an aromatic divinyl hydrocarbon, said copolymer having quaternary ammonium groups having hydrocarbon groups as nitrogen substituents attached to the aromatic nuclei thereof through an alkylene linkage of 1 to 4 carbon atoms and having heparin ions as anions, thereof, said copolymer containing at least 1 of said groups for each 15 aromatic nuclei.

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